AN INTRODUCTION TO PHYTOCHEMICAL METHODOLOGY

WITH SPECIAL REFERENCE TO FLAVONOIDS

Earth is a planet dominated by plants. The green plant is fundamental to all other life. The oxygen we breathe, the nutrients we consume, the fuels we burn and many of the most important materials we use were produced by plants.

Plants represent the first stage in the evolution of living things. In the process of the growth of nature, plants multiplied in number, variety and types. Humanity has identified as many as 7.5 lakhs species of plants\(^1\) on earth, of which 5 lakhs are classified as “higher plants” and 2.5 lakhs as “lower plants”.

The association between plant and man is an age-old process starting from human civilization. There has always been a race between nature and human knowledge. The plants sustain nature and nature sustains them. The interdependence of man and nature increases day by day. If human race makes sensible use of nature, posterity is bound to be prosperous.

Since their evolution, plants became primarily useful for mankind. Realization of the importance of plants in the welfare of humanity prompted their systematic study from different angles. Thus many plant species\(^6,7\) have been subjected to detailed scientific analysis over the years. Yet not more than 10% of all the plants could be analyzed and a vast majority still remains uninvestigated. The study continues in various fields and provides challenges to several groups of plant scientists.

India with its vast area from Kashmir to Kanyakumari and varying soil and climatic conditions ranging from tropical to temperate has one of the world’s richest
vegetations, comprising of about 1,30,000 species of plants belonging to 120 families to be aptly called "the Botanic Garden of the World." India has a rich heritage of indigenous drugs from the Vedic times. The Ayurvedic system of medicine is purely of Indian origin and development. More than 2400 remedies have been known from Indian medicinal flora. The remarkable properties and therapeutic uses of about 700 plant drugs have been recorded by ancient Indian scholars, vrz. Sushrutha, charaka and vagbhatta before 1000 B.C. Sanskrit literature written by them contains information about morphological features of many medicinal plants, their geographical distribution condition of growth, the best season for their maximum potency as well as their toxic properties.

Another ancient system of medicine flourished in south India is the siddha system. This system was introduced by the siddhars. They were those who attained or achieved perfection or heavenly bliss. They were the greatest scientists of ancient times and men of culture, intellectual and spiritual faculties combined with super natural powers.

Among siddhars, there were eighteen Maha siddhars, viz, Nandhi, Agasthia, Mular, Punnakesar, Pulasthiar, Punaiykanar, Idaikkadar, Bohar, Pulikairesar, karurar, konganavar, kalangi, Azukannar, Agappiar, Pampatti, Theraiar, Kudambaiyar, Sattainathar. They imparted in different periods of history, not only their knowledge of medicinal plants but also their philosophy to their students and successors.

According to siddhars school, the human body is composed of 96 tatwas, 72,000 blood vessels, 13,000 nerves, 10 main arteries and 10 vital airs (prana) in the form of a network. It is owing to the derangement of the three humours, the body becomes liable
to 4448 diseases. There are medicinal plants in India to cure all the above diseases of mankind (sage Agasthya)\textsuperscript{14}.

These siddhars did not only cure the dreadful diseases like cancer, leprosy etc. by simple herbal powders, but also attained the immortality by a plant medicine popularly called by them as "Muppu".

According to them "Muppu" is composed \textsuperscript{18} of three salts, separated from a medicinal plant and reunited, which rejuvenate the human body. Rejuvenation does not necessarily mean restoring youthfulness to the old, but it simply means the maintenance of youth without reaching old age, if youth is maintained perpetually it becomes immortality.

India like all other countries has made significant progress by a systematic scientific study of these plant drugs from the pharmacological, chemical, pharmacognostical and clinical points of view during the past 65 years. This brought to the forefront a large number of herbs used in Indian indigenous system for their approved efficiency and administration in modern medicines.

Recognizing the importance of medicinal plants collaborative team work for a complete study of plant drugs has been encouraged by the Indian Council of Medical Research (ICMR) Central Council for Research in Ayurveda and Siddha (CCRAS) and council for scientific and Industrial Research (CSIR). The results of various studies on Indian medicinal plants are available\textsuperscript{26-37}. Central Drug Research Institute (CDRI) Lucknow initiated a programme from 1979 to investigate Indian plants which either had a reputation in folk medicine or whose extracts showed consistent biological activity
when put to a broad biological screen. So far under this programme over 8000 species were screened and biological activity confirmed in about 650 plants.

Thorough investigation of siddhar's literature reveals that each and every plant contains both poisonous chemicals as well as medicinally useful chemicals. The medicinal property of the plant alone was retained, by treating this plant with other simple chemicals or some chemical methods to neutralize the poisonous effect of the plant. Moreover siddhars have used a mixture of herbal powders in some fixed ratio than a single herbal powder.

The above studies provide further scope for undertaking research work in several directions. The phytochemicals both pharmacologically active and poisonous and their structural changes can be discovered. Besides, there are different results regarding the structural modifications of pharmacologically active phytochemicals obtainable by combining different herbal powders at various proportions. If the modern methods of investigation of medicinal plants in India with the newly developed scientific approach are applied, this type of research will provide for the discovery of new effective plant drugs.

Phytochemistry, evolved from natural products chemistry is confined to the study of products elaborated by plants and it has developed as a distinct discipline between natural product organic chemistry and plant biochemistry in recent years. It deals with the study of chemical structures of plant constituents, their biosynthesis, metabolism, natural distribution and biological functions. The fact that only less than 10% of about 7.5 lakhs species of plants on earth has been investigated indicates the opportunity provided and challenges thrown open to phytochemists. The task of the
phytochemist is compounded in accomplishing the characterization of very small quantity of the compounds isolable from plants. Phytochemistry also enjoys the application of modern research for the scientific investigation of ancestral empirical knowledge. It has found wide and varying application in about all fields of life and civilization. Its direct involvement in the field of food and nutrition, agriculture medicine and cosmetics, is well known for years. Its contribution even in seemingly remote areas such as plant physiology, plant pathology, plant ecology, palaeobotany, plant genetics, plant systematics and plant evolution has been increasingly felt. One of the more encouraging trends as phytochemistry continues to grow and develop as a scientific discipline is the wider and wider applications that are occurring in agriculture, horticulture and forestry.

Among the phytochemicals, the polyphenolics constitute a distinct group. They embrace a wide range of substances, which possess in common an aromatic ring bearing one or more hydroxy substituents or their ether or glycoside derivatives. These compounds possess great structural diversity and are of widespread occurrence among the secondary metabolites. A further feature of this particular group of compounds is their ability to interact with primary metabolites such as polysaccharides and proteins.

Among the several thousands of naturally occurring poly phenolic compounds, the flavonoids are the largest and the most widespread. The term “flavonoid” was first applied about 48 years ago by Geissman and Hinreiner to embrace all those compounds whose structure is based on that of flavone (2-phenyl chromone) (I) having basic C₆ - C₃ - C₆ skeleton in common when the heterocyclic ring is reduced, it becomes flavan (2-phenyl chroman) (II), Flavone (I) consists of two benzene rings (A and B)
joined together by a γ - ring (ring C). The various classes of flavonoid compounds differ from one another only by the state of oxidation of this carbon link. There is a limitation to the number of structures commonly found in nature, which vary in their state of oxidation from flavan 3-ols (catechin) (III) to flavonols (3- hydroxy flavones) (IV) and anthocyanins (V). Flavanones (VI), flavanonols or dihydro flavonols (VII) and the flavan 3-4-diols (proanthocyanidins) (VIII) are also included in the flavonoids. It should be noted that there are also five classes of compounds (dihydro chalcones or 3-phenyl propiophenones (IX) chalcones or phenyl styryl ketones (X) isoflavones or 3-phenyl chromones (XI) neoflavones or 4-phenyl coumarins (XII) and the aurones or 2-benzylidine-3-coumaranones (XIII) which do not actually possess the basic 2-phenyl chromone (I) skeleton, but are closely related both chemically and biosynthetically to other flavonoid types, that they are always included in the flavonoid group.

The individual compounds in each class are distinguished mainly by the number and orientation of hydroxy and methoxy groups in the two benzene rings. These groups are usually arranged, reflecting the different biosynthetic origin of the two aromatic nuclei. Thus, in the A ring (I) of the majority of flavonoid compounds, hydroxy groups are distributed at either C-5 or C-7 or only at C-7 (C-5 and C-7 of flavone become C-2' and C-6' in dihydrochalcones and chalcones and C-4 and C-6 in aurones) and generally are unmethylated. This pattern of hydroxylation follows from the acetate or malonate origin of the ring. The B-ring (I) of flavonoids on the other hand is usually substituted either by one, two or three hydroxy or methoxy groups. The rarely methylated position is C-4' with often methylation at C-3' and C-5'. The hydroxylation
pattern of the B-ring thus resembles that found in commonly occurring cinnamic acid (XIV) and coumarins (XV) and reflects their common biosynthetic origin from prephenic acid and its congeners.
STRUCTURES OF SOME COMMON FLAVONOID TYPES

I Flavone

II Flavan

III Flavan 3-ol

IV Flavonol

V Anthocyanidin

VI Flavanone

VII Dihydroflavonol

VIII Flavan 3,4-diol
IX Dihydrochalcone

X Chalcone

XI Isoflavone

XII Neoisoflavone

XIII Aurone

XIV Cinnamic acid

XV Coumarin
A comparison of the nature and position of various substituents at different carbons of the flavonoid skeleton has been made use of in arriving at certain generalizations regarding structure and properties. Most of the flavonoids occur naturally in conjugated form, usually bound to sugar, by a hemiacetal linkage. But their conjugation with inorganic sulfates or organic acids is not unusual. The sugar free compounds are referred to as aglycones and it is probable that in most cases they are formed as artefacts during the course of extraction, since most living tissues contain very active glycosides which can work even in the presence of high concentration of organic solvents. The presence of sugars in the molecule confers sap-solubility to the generally some what insoluble flavonoid compounds. In anthocyanins the sugar imparts stability to the aglycone. Stability conferred by glycosylation to flavonols is observed in 3-O-glycosides of quercetin and myricetin, which are not susceptible to oxidation catalysed by phenolase unlike the corresponding aglycones, presumably because of steric reasons. More and more range of new glycosides are encountered in plants. An increasing number of flavonoid glycosides carrying sugars in B-ring hydroxyls have been reported. Conjugation of flavones and flavonols through glucose with organic acids like malonic acid and derivatives of cinnamic acids have also been reported. The number of acylated flavonoid compounds succeeded by terpenoid counterparts is on the increase. As a result of electrophoretic studies, a number of zwitter ionic anthocyanins with malonic acid and succinic acid linked to C-6 of glucose have been isolated and characterized.

The sugars found in flavonoid glycosides include simple pentoses and hexoses (monosides) and di-and tri-saccharides (biosides and triosides) mostly combined
through oxygen at C-1 position of sugars, usually by a β- linkage. In many cases, more than one phenolic hydroxyl group in the flavonoid molecule may be glycosylated giving rise to diglycosides and so on. The common sugars are D-glucose, D-galactose, L-rhamnose, D-xylose, L-arabinose and D-glucuronic acid. D-allose and D-galacturonic acid are rare and D-apiose is an unusual and uncommon one.

The study of the distribution of flavonoids in plants\textsuperscript{74,75} is continuing exercise and known flavonoids are being regularly discovered from new sources. Flavonoids are universal in vascular plants, but variation according to phyla, order, family and populational variation with in species have been detailed by Harborne and Turner\textsuperscript{76}. The presence of flavonoids in butterflies has been recognised\textsuperscript{77} in 1983. In 1985, the distribution of flavonoids in animals has been reported with the isolation of 4'-methoxy flavone from scent glands of Canadian beaver (castor feber) and in Lepidoptera\textsuperscript{78}.

Standard methods of extraction, separation and chemical characterization of flavonoid compounds are described by Tracey\textsuperscript{79} as well as Harborne\textsuperscript{42}.

Systematic procedure for the flavonoid identification employing chromatographic methods of analysis and chemical and spectral methods of identification have been explained by Geissman\textsuperscript{46}, Harborne\textsuperscript{67}, Mabry et al\textsuperscript{80}, Jay et al\textsuperscript{81}, Markham\textsuperscript{82} and Linskens and Jackson\textsuperscript{83}.

The conventional chromatographic methods like column, paper and thin layer are still in use for separation and purification of the flavonoid compounds. Increase in speed and efficiency in the separation of mixtures had been achieved by high pressure liquid chromatography (HPLC). Among the separation techniques applied to flavonoids
HPLC has the advantage over to other techniques in regard to sensitivity, rapidity and easy quantification.

Hostetttman and Hostetttman reviewed the relevant literature on HPLC up to 1980. A few other publications in this field include Rt values by Daigle and Conkeston, use of Bondpak C\textsubscript{18} with MeOH-HOAC-H\textsubscript{2}O as developing system with two pumps by casteele et al, analytical problems in HPLC by Bankova et al, Tamma et al and Barberan et al. The application of HPLC combined with FABMS in the structural elucidation of anthocyanin pigments was explained by J.B.Harborne and R.J.Grayer. On line HPLC-UV, flash chromatography, centrifugal TLC using chromatotron are also applied in the isolation of flavonoids. For difficult separations requiring very high resolution semi preparatory HPLC with automatic fraction collector is an ideal method. Reverse phase chromatography, HPLC-DAD and HPLC-MS on chemically bonded phases gives better results of the separation of plant phenolics.

The complication of irreversible adsorption and decomposition of the solute at the liquid-solid interface in all techniques employing a solid stationary phase and liquid mobile phase is overcome by various support free liquid-liquid partition techniques, DCCC and RLCC have been employed profitably for quantitative and qualitative separation by Hostetttman and collaborators, Aritani et al and Gunasegaran et al. However, the constant need in natural product chemistry to separate large and small quantities of complex mixtures efficiently and rapidly is unfortunately seldom satisfied by the use of any one chromatographic technique. The
best results have been obtained by a combination of several techniques, which are often complementary.

Paper electrophoresis\textsuperscript{108} is a technique of limited application in flavonoid analysis, since to be mobile, a flavonoid must be in an ionized state at the pH of the electrolyte. Its useful application lies in the recognition and identification of flavonoid sulfates\textsuperscript{109,110} and in the distinction of glycuronides from glycosides\textsuperscript{111}. Relative mobilities of different flavonoid sulfates are listed by Hostetman\textsuperscript{105}. Electrophoresis finds greater application in the field of anthocyanins and betacyanins.

The flavonoid once isolated as a homogenous compound is characterized by the specific colour tests\textsuperscript{52}, physical constants\textsuperscript{53}, elemental analysis\textsuperscript{54}, $R_f$ values\textsuperscript{55} in various solvent systems, analysis of hydrolysis products, preparation of derivatives and comparison of these data with related compounds\textsuperscript{75}. Further support for confirmation of the structure of the flavonoid is achieved by the analysis of different spectral data (UV-VIS, IR, MS, $^1$H and $^{13}$C NMR).

Ultraviolet spectroscopy\textsuperscript{80} is still one of the oldest and useful techniques for flavonoid identification. The UV spectrum with MeOH and with diagnostic shift reagents can give information on the type of flavonoid as well as its substitution pattern. The use of AlCl$_3$ and AlCl$_3$/HCl UV spectra in the precise determination of the structure of flavonoid compound was demonstrated by voirin\textsuperscript{112} and the limitation of NaOAc spectra in flavonoid analysis has been reported by Rosler et al\textsuperscript{113}.

IR spectroscopy\textsuperscript{114} provides valuable information regarding the type of flavonoid as well as the nature of the substituents like methoxy, methylene dioxy and prenyl oxy
groups. It is also used as a "finger-printing" devise for establishing the identity of two samples.

Mass spectrometry of flavonoids serves as a valuable aid in determining the molecular weight and probable structure even with very small sample size. The electron impact mass spectrometry (EIMS)\textsuperscript{115} is used for the volatile compounds, the non volatile compounds are converted to suitable derivatives like trimethyl silyl ether, permethyl ether, methyl ester or similar derivatives. Field desorption mass spectrometry (FD - MS) employed for polar and thermolabile compounds like flavonoids has been reviewed by Schulten and Games\textsuperscript{116}. Desorption chemical ionization mass spectrometry (DCI - MS)\textsuperscript{117} using electrically heated tungsten probe and fast atom bombardment mass spectroscopy (FABMS)\textsuperscript{118} using the sample solubilised in polar matrix (like glycerol, thioglycerol etc.,) deposited in a copper target, which is bombarded with energized neutral atoms to induce ionization and desorption\textsuperscript{119} appears to be the most advantageous one for the analysis of flavonol glycosides, when two MS are linked in tandem, it has now become possible to employ the first as separator and the second as analyzer to perform direct mixture analysis (Tandem-MS). This multiple stage MS has been reviewed by Roush et al\textsuperscript{120}. In the chemical ionization mass spectrometry (CI - MS)\textsuperscript{121} the ions are formed in ion molecular collisions which include abstraction as primary process using weak gas phases like CH\textsubscript{4}, NH\textsubscript{3}, i- C4 H10 in positive CI for protonation. In negative CI, OMe\textsuperscript{-} as reagent for proton abstraction or Cl\textsuperscript{-} as an attachment reagent is employed. The MS analysis of permethyl ether of glycosides is widely employed for settling structural problems in C - glycosyl flavones \textsuperscript{122, 123}.
Pyrolysis chemical ionization mass spectrometry of flavonoids under positive and negative ionization conditions is observed\textsuperscript{124} to yield data characteristic of both aglycone and sugar residues, providing an alternative for FD and FABMS techniques. The easy MS differentiation of flavanones and dihydro flavonols by characteristic fragments has been reported\textsuperscript{125}. The application of GC–MS\textsuperscript{126} analysis to perdeuteromethylated derivatives of flavonoids\textsuperscript{127} has rendered the identification of certain methoxylated compounds easier. On line HPLC–MS\textsuperscript{128} will be readily accepted by flavonoid researchers. Becchi and Fraisse\textsuperscript{129} have reported mass analysed ion kinetic energy (MIKE) and collision activated dissociation. MIKE spectra of flavonoids providing characteristic fragment ions, which permit differentiation of the 6– and 7– or 8– substituent location and the position of O–glycosylation. Solution phases secondary ion mass spectrometry\textsuperscript{130} has proved useful in the determination of molecular weight of complex flavonoid glycosides. Electrospray MS\textsuperscript{114} has become quite versatile on account of the amanability of the technique to highly polar and large sized molecules including biomolecules like proteins Recently ESI and MALDI – TOF mass spectrometry in the study of flavonoids and selected components of biological interest have been used\textsuperscript{131,132}.

Nuclear Magnetic Resonance (NMR) over the last twenty years has given considerable encouragement to structural elucidation in all fields of natural product chemistry. The proton magnetic resonance spectroscopy is the established non destructive method of flavonoid analysis. Use of high field magnets and computer assistance has made the recording of high resolution $^1$H NMR spectra of minute quantity of flavonoids\textsuperscript{133-136}. Typical application of $^1$H NMR includes determination of
oxygenation pattern, number of methoxy group, distinction of isoflavones, flavonones and dihydroflavonols, number and nature of sugar present (whether $\alpha$-linked or $\beta$-linked) and detection of hydrocarbon side chain. Shift reagent provide a method of spreading out PMR absorption signals. The use of lanthanide shift reagents in positioning of methoxy groups of flavonoids was reported by Joseph Nathan and et al.$^{133}$

Of late $^{13}$C NMR spectroscopy$^{137-142}$ has become the most useful technique for the structural determination of flavonoids. $^{13}$C resonance signals extending over 200 ppm provide the nature of the carbon skeleton. Carbon relaxation time measurement being less difficult is very useful in differentiating otherwise non discernable carbon atoms like C-6 and C-8 in flavonoids. Inter glycosidic linkages in the case of disaccharides$^{143}$ (rutinose, neohesperidose etc.,) type of linkage with aglycone and sugars and conjugation with sulfates and organic acids can also be determined. Homonuclear and heteronuclear correlation spectroscopy$^{144}$ (HOMCOR and HETCOR) and the various decoupling experiments have reduced the difficulty in the interpretation of $^1$H and $^{13}$C NMR spectra of complex molecules. Fourier transform NMR employing popular pulse sequences like off – resonance, heteronuclear decoupling, gated heteronuclear decoupling, inverse gated heteronuclear decoupling and selective proton decoupling has eased the task of assignment of peaks in a $^{13}$C NMR spectrum. Refocussed INEPT with decoupling is an alternate of off-resonance decoupling for assigning carbon multiplets. J-modulated spin echo$^{145}$spectroscopy is yet another method of off–resonance decoupling which gives positive signal for carbon with even
number of hydrogen (CH\textsubscript{2} and quartenary) and negative signal for carbon with odd number of hydrogen (CH and CH\textsubscript{3}) attached. At present 2D NMR\textsuperscript{146,147} methods make it more acceptable to natural product chemistry with the requirement of decreased sample size usually a set of routine 2D experiments\textsuperscript{148} including COSY (and / or DQF COSY), relayed COSY [(HOHAHA), 2D NOE (NOESY / ROESY) and HETCOR (HMQC)] are used in the structural elucidation of flavonoids. Recently NMR techniques have been extended to clarify the anti oxidative molecular mechanism of catechins\textsuperscript{149}.

Over the past 40 years, chiroptical methods like ORD and CD spectral analysis have been employed for the determination of stereochemistry of chiral flavonoids\textsuperscript{150,151}. The exciton chirality method\textsuperscript{152} employing the application of coupled oscillators in determining the chirality of natural products is receiving greater attention.

If the flavonoid compounds can be obtained in a fine crystalline form, X-ray analysis can help in further confirming the structure as reported in the case of calycoperin\textsuperscript{153}.

Final confirmation is always desired to be established by unequivocal total synthesis. The conventional methods of synthesis of flavonoids from simple precursors by condensation methods have been proposed by Algar and Robinson\textsuperscript{154}, Algar and Flynn\textsuperscript{155} and Baker and Venkataraman\textsuperscript{156}. These methods of synthesis have been modified by Farkas et al\textsuperscript{157} and Wagner et al\textsuperscript{158} as illustrated by the synthesis of a number of flavonoids and their methyl ethers. Many flavonoid compounds have been prepared by simple modification of the existing structure through nuclear oxidation, nuclear reduction, isomerisation, selective alkylation and dealkylation, selective glycosylation and partial hydrolysis. Wagner et al\textsuperscript{158} accomplished the synthesis of
methoxylated flavones from the corresponding brominated methoxy chalcones. The synthesis of 5,6,7,3',4'-penta methoxy flavone (sinensetin) by dehydrogenation of the corresponding flavonone with SeO₂ has been achieved by Wagner et al. This flavone has been used as the starting material for the synthesis of a number of related flavones. Bose et al. have reported cyclisation and simultaneous dehydration of the hydroxy chalcone to the corresponding flavone by heating with palladium on charcoal. A short and facile synthetic route to hydroxylated flavones has been reported by Nagarathnam and Cushman.

The synthesis of flavonoid glycosides have been achieved using the α-acetobromosugars of pentoses, hexoses or disaccharides and the aglycones in the presence of catalysts. The selective glycosylation of 7-OH has been achieved by Zemplen and Farkas. Synthesis of other glycosides have been accomplished by transacylation methods by Nogradi et al. and Wagner et al. The total synthesis of C-glycosyl flavones has been reported by Eade et al. and other complex ones has been provided by later workers. The chiron approach to the total synthesis of natural products might become a useful guide in the synthesis of Chiral flavonoid compounds. Thus the synthesis of almost all types of mono and di-C-glycosyl flavones and flavone C-glycosyl-O-glycosides has been accomplished, synthesis of some novel flavonoids has been illustrated by Rakosi et al. Studies of the selective O-alkylation and dealkylation of flavonoids with anhydrous AlBr₃ were reported by Horie et al.
Our present knowledge in flavonoid biosynthesis is based on a combination of earlier results from radioactive tracer studies in vivo and the more recent data obtained at the enzyme level in vitro. In the past few years the enzymology of flavonoid biosynthesis has made particularly rapid progress. Flavonoid biosynthesis can be considered in three stages. The first stage is the formation of the basic $C_6-C_3-C_6$ skeleton through acetate-malonate and shikimic acid pathway to aromatic compounds. The second stage is concerned with the ways by which the different classes of flavonoids are synthesized. The final stage embraces the elaboration of individual compounds with in each flavonoid class, involving steps such as hydroxylation, glycosylation, methylation etc. Chalcone considered as common intermediate in the biosynthesis of all classes of flavonoids. Insight into three aspects of the problem of flavonoid biosynthesis has come in the past from comparative anatomy, chemical genetic studies and recently from feeding experiments with radioactive tracers.

Research has led to the isolation and characterization of enzymes of the pathway of biosynthesis. The use of young plant tissues and cell suspension cultures as source materials have also greatly facilitated the study of flavonoid biosynthesis at the enzyme level. Roux and Ferreira have highlighted the special role of $\alpha$-hydroxy chalcone as the key intermediate in flavonoid biogenesis. A comprehensive report on the biosynthesis of flavonoids by Hahlbrock et al., Wong, Birch et al., a good account of biosynthesis of shikimate derived phenolic compounds by Harborne, Manitto, biosynthetic studies in vivo with labeled precursors and biochemistry of flavonoids biosynthesis by Heller and Heller and Forkman are useful publications. Recent
trends in the biosynthesis of flavonoids have been discussed by Akashi et al\textsuperscript{188}, Bennett et al\textsuperscript{189}, Kennedy et al\textsuperscript{190} and Fedoreyev et al\textsuperscript{191}.

The importance of flavonoids and other secondary metabolites in plant biochemistry has been detailed in "the biochemistry of plants"\textsuperscript{192}. Different aspects of mammalian metabolism of flavonoids have been reviewed by De Eds\textsuperscript{193}, Scheline\textsuperscript{194}, Griffiths\textsuperscript{195}, Middleton Jr and Kandaswami\textsuperscript{175} and Bohm\textsuperscript{196}. Flavonoids are the constituents of the mammalian diet derived from plants. The ingestion of flavonoids by mammals in the diet or for therapeutic use, brings them in contact with both intestinal microorganisms and mammalian tissues which are capable of bio transformation of flavonoid compounds. The available evidences\textsuperscript{197} indicate that the hydrolysis of the flavonoid glycosides to their corresponding aglycones, ring fission and oxidative and reductive transformations are mediated by intestinal microorganisms. Though it is certain that the metabolic changes undergone by flavonoids occur with in mammalian tissues, the relative contribution of individual tissues is not fully understood.

The flavonoids find exceptional use as chemotaxonomic guide in the classification of plants. The reason for preferring flavonol to other secondary metabolites is their structural diversity, widespread distribution, comparative stability, easy detection and identification and the fact that this group of plant products is not actively concerned with cellular metabolic processes. Any particular flavonoid can be relied to be present in more or less constant amount in the same tissue of the same species so long as the plants are grown under normal physiological conditions. The conspicuous exception to this is the variation in the relative concentration of p-coumaric acid and caffeic acid (mono and dihydroxy phenyl propanoid precursors) as well as
kaempferol and quercetin which are insignificant from the chemotaxonomic point of view. Importance of flavonoids in chemotaxonomy can be illustrated with a few examples. A chemosystematic study on Euphorbiaceous\textsuperscript{198} plants was performed using polyphenolic constituents. The phenolic characteristics of sub families genera and species were well distinguished from one another. Hydrolyzable tannins as constituents were considered to be a valuable chemotaxonomic character in elucidating systematic relationships among the related taxa. \textit{Ziziphus jujuba} and \textit{Ziziphus vulgaris} of Rhamnaceae have been differentiated chemotaxonomically based on juzacic acid and betulinic acid\textsuperscript{199}. Recently 3,4 –dimethoxy cinnamic acid levels as a tool for the chemotaxonomic differentiation of 13 species of \textit{Coffea canephora} var \textit{robusta} and 7 species \textit{Coffea arabica} of Rubiaceae family have been studied\textsuperscript{200}. Further the occurrence of flavonols and mannitol\textsuperscript{201} in the species of Rubiaceae can also be used as a chemotaxonomic marker of the family. Among the numerous flavonoids of higher plants the rare and unusual ones find more acceptance in micromolecular taxonomy. Recently chemotaxonomic markers in \textit{Calophyllum teysmannii}\textsuperscript{202} of Guttiferae, \textit{Bursera species}\textsuperscript{203} of Bureseraceae and other plants\textsuperscript{204-206} have been extensively studied.

The most significant function\textsuperscript{207-210} of the sap-soluble flavonoids is their ability to impart colour to the plants in which they occur. They are responsible for most orange, scarlet, crimson, mauve, violet and blue colours, as well as contributing much to yellow, ivory and cream flowers. The only other considerable groups of colouring matters in higher plants are the lipid-soluble chlorophylls and carotenoids. Chlorophylls-a and b provide the prevailing green of plant leaves, and the carotenoids are the most important sources of yellow and orange colours in flowers and fruits.
The importance of anthocyanin\textsuperscript{211-217} colour in fruits such as the strawberry, cherry, black currant and so on as an aid to seed dispersal by animals is self-evident. Both fruit and flower colour give immense aesthetic pleasure to man and conscious selection for colour varieties among garden plants and horticultural crops has been practiced for a very long time. Several physiological functions for anthocyanin in the general metabolism of plants described in the literature\textsuperscript{218, 219} are still rather obscure. Anthocyanins may also be important factors with other flavonoids\textsuperscript{219} in the resistance of plant in insect attack. Several speculations about the role of anthocyanins on the perception or filtration of light and response to stress factors, including microbial attack await further thorough studies.\textsuperscript{220, 221} Recently anthocyanins as food colours has been discussed by Gonnet.\textsuperscript{222}

The important function of flavonoids in plants are their protective role as light screen against damaging UV radiation, as feeding deterrents and protection from herbivores and as allelopathic agents. In biological systems, the stimulation of protein degradation as well as protein formation by antibiotics resulted in flavonoid accumulation implicating the importance of flavonoids in protein synthesis.\textsuperscript{223, 224} Other important functions include their role as anti-oxidants, enzyme inhibitors, precursors of toxic substances and as photosensitizing and energy transferring compounds, in control of plant growth and development, in respiration, photosynthesis, morphogenesis and sex determination in plants. In general flavonoids show potential as environmentally less harmful insecticides, especially when applied systematically, and knowledge of their properties in plants to deter insects can be used to breed more resistant crops. Thus, if flavonoids are used in integrated pest strategies, and as long as
agronomists and plant breeders are aware of the properties of these compounds and other classes of secondary substances, they have a real potential for crop protection in the future.

Apart from the physical and morphological means, chemical means are a major method of plant defence. The introduction of isoflavonoid phytoalexins in plant causes phyto toxicity such as inhibition of respiration, reduced growth of suspension cultures, repressed seed germination, retarded root growth and electrolytic leakage. Different types of bioassay involving several types of organisms conducted with isoflavonoids, phytoalexins by Smith revealed that they possessed fungi toxicity and limited antibacterial activity. A significant range of flavonoids have been encountered as anti fungal agents. A number of flavonoids are being induced in plants following fungal invasion. Some of the flavonoids affect the behaviour, growth and development of insects due to their toxicity, while flavone glycosides are feeding stimulants. The metabolic challenges of the plant flavonoids including the condensed tannins to the insects consists mainly of the variety of phenolic groups. This activity is destroyed when the phenolic OH group is methylated in flavonoids. The structure – activity effects have been studied for a number of flavonoids for anti – growth and anti – bacterial activity and it has been found that growth inhibiting activity depends on the presence of ortho dihydroxy group in ring A and B and not the functional group of ring C and the position of ring B (at C – 2 or C – 3 as in flavones or isoflavones). The glycosylation of flavonoids also show marked variation in growth inhibition; 3-O-glycosylation inhibits growth but not 7-O-glycosylation. The enhanced activity of the flavonones compared to
the corresponding flavones showed that the co-planarity of the flavonoid rings of flavones might hinder their biological efficacy.

An ever-increasing number of pharmacological effects of flavonoids have become known over the years through the discovery of new plant flavonoid and their derivatives. The anticancer, anti-viral, anti-oxidant, anti-microbial, and anti-inflammatory effects of flavonoids are noteworthy. In recent years a number of flavonoids like baicalin, taxifolin, gossypin, proanthocyanidins, nepetin, diosmin, fisetinsophoricoside, (+)-catechin, (-)-epicatechin and 5,7-dimethoxyflavone have been reported to have anti-inflammatory effects.

The effects of a few flavonoids on different types of viruses were investigated by Pusztai et al. and observed that hydroxylation in 3-position appears to be a prerequisite for this activity. Anti cancer activity of a few flavonoids (flavones, isoflavones, flavonones and flavonols) are reported. Anti-rhinovirus and anti picronovirus activity of certain flavonoids have also been reported. The effect of many semi synthetic flavonoid derivatives on arachidonate metabolism was established of which (O-β-hydroxy ethyl) rutin and various quercetin derivatives are important. The effect of naturally occurring flavonoids on this metabolism was also reported by Ferrandiz et al. Studies of compounds with Flavone skeleton were stimulated by recognition of anti-allergic effects. Thus, orally effective anti-allergic chromone drugs (Kellin, hypolaetin-8-glycoside disodium chromoglycoside etc) are in use. Recently 7,8-di-O-substituted flavans, biflavans and flavones showed cytotoxic activity and it has been established that the activity is due to methoxy and/or hydroxy group in the structure.
This screening of plants, recorded in Indian folk medicines, for liver injury has established\textsuperscript{245} that the active principles are flavonoids. The anti-hepatotoxic effect of flavonoids was first demonstrated by Halm et al\textsuperscript{246} with the flavanolignan, silybin and its isomers. About 13 flavonoids and coumarins have been demonstrated\textsuperscript{247} to be anti-hepatotoxic or liver protective agents.

Anti-ulcerogenic property of 3-O-methyl (+)-catechin, apigenin and luteolin, anti-diabetic effects of hispidulin and nepetin and analgesic effects of puerarin are also reported\textsuperscript{248,249}. Recently herbal remedies for skin diseases\textsuperscript{250-252} obtained from plants used in Unani system of medicine are useful publications. Inhibition of human immunodeficiency virus reverse transcriptase is currently considered a useful approach in the prophylaxis and intervention of Acquired Immunodeficiency syndrome (AIDS) and natural products have been extensively explored as inhibitors of this enzyme, to discover drugs active against AIDS. One hundred and fifty pure natural products have been examined\textsuperscript{253} and polyphenolic compounds were found to be responsible for the activity, among flavonoids tested quercetin exhibited moderate activity.

Work on different aspects of flavonoids including discovery of new compounds, biological activity, medicinal properties etc., are reviewed regularly\textsuperscript{50-56} and the knowledge is updated through proceedings of regular international symposia on flavonoids\textsuperscript{254}. Considerable work on the chemistry and pharmacological properties of a number of flavonoids has been accomplished in the laboratories of our Institution and university\textsuperscript{255,267}. 
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